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Techniques for starch gel electrophoresis of enzymes from forest tree species

W.M. Cheliak and J.A. Pitel

Information Report PI-X-42
Petawawa National Forestry Institute

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PETAWAWA NATIONAL FORESTRY INSTITUTE

The Petawawa National Forestry Institute (PNFI) was formed on April 1, 1979, as the result of an amalgamation of the Petawawa Forest Experiment Station with the Ottawa-based Forest Management and Forest Fire Research Institutes. The Forestry Statistics and Systems Branch was established at PNFI in 1980.

In common with the rest of the Canadian Forestry Service, the Petawawa National Forestry Institute has as its objective the promotion of better management and wiser use of Canada's forest resource to the economic and social benefit of all Canadians. Because it is a national institute, particular emphasis is placed on problems that transcend regional boundaries or that require special expertise and expensive equipment that cannot be duplicated in CFS regional establishments. Such research is often performed in close cooperation with staff of the regional centres or provincial forest services.

Research at the Institute is in two main areas:

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The operations of PNFI also include THE FORESTRY STATISTICS AND SYSTEMS BRANCH (FSSB) which is responsible for the acquisition and publication of national information on the forests of Canada. Through the Canadian Forest Inventory Committee, which is comprised of provincial and federal forestry officials, the FSSB works in close cooperation with provincial forest agencies to improve and standardize the information available on Canada's forest resources.

Through the FORSTATS program, which involves all regional establishments of the Canadian Forestry Service, the FSSB coordinates the acquisition and publication within the CFS of national statistics on the forest of Canada.

Every five years, the FSSB publishes Canada's Forest Inventory; the official report on the location, extent, species, and condition of the forest resource. In addition, the FSSB is working closely with the provinces to expand the information available on changes to the forest from fire, harvesting, insects and disease, and from forest management activities. This information is essential to the development of sound policies for the improved management of this important and renewable natural resource.

TECHNIQUES FOR STARCH GEL ELECTROPHORESIS OF ENZYMES FROM FOREST TREE SPECIES

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Abstract

Methods are presented which fully describe the process of protein starch gel electrophoresis for forest tree species. Specific sections deal with procedures for haploid tissues (megagametophytes from conifers) and diploid tissues. The diploid tissues discussed include embryo, leaf, bud, needle, and bark tissues of both conifer and angiosperm species. Several appendices outline extraction, electrode, and gel buffers, and enzyme staining recipes used for forest tree species. A bibliography is included that allows access to literature dealing with applications of electrophoresis to the study of forest tree populations.

Résumé

On présente le mode opératoire détaillé de l'électrophorèse des protéines d'essences forestières en gel d'amidon. Des parties précises de la publication traitent de la manipulation des tissus haploïdes (mégagamétophytes des conifères) et des tissus diploïdes (embryons, feuilles, bourgeons, aiguilles et tissus des conifères et des angiospermes). Dans plusieurs annexes on donne, dans leurs grandes lignes, des renseignements sur l'extraction, les électrodes, les solutions tampons ainsi que des recettes de coloration des enzymes pour les différentes essences forestières. Une bibliographie permet d'accéder aux publications traitant des applications de l'électrophorèse à l'étude des populations d'arbres forestiers.

PREFACE

This manual is written in order to share with others some experience we have gained in the electrophoresis of tree tissues. We hope the manual will be of assistance to those just starting an electrophoresis laboratory, and also to others who are familiar with the techniques but are just initiating work with trees. Since our laboratory operates within a regular working schedule, it is necessary to accommodate the routine of analysis into an eight hour working day. Some refinements presented represent our solution to this problem.

Enzyme stains are expensive; therefore, the more slices of gel stained per day per enzyme system the less is the total cost of an experiment. With these considerations in mind, it is hoped that the manual will be of benefit in allowing more extensive experiments to be conducted without additional time or cost.

No two labs can be operated in exactly the same way because lab personnel, equipment, and facilities vary. Therefore, the primary purpose of this manual is to describe general principles, rather than specific procedures.

A bibliography of selected references is presented in Appendix III. It has been organized to allow efficient access to a large body of literature.

TECHNIQUES FOR STARCH GEL ELECTROPHORESIS OF ENZYMES FROM FOREST TREE SPECIES

1 INTRODUCTION

The study of quantitative characters for crop improvement has been, and remains, the essential basis for phenotypic selection in a breeding program. However, the ability of these characters to provide measures of genetic variation within, and estimates of genetic similarity among, populations is limited by time and precision of genetic expression. Commonly, the time required for expression of characters related to yield amounts to years when working with forest tree species. In addition, phenotypic characters are subject to environmental modification and many are polygenic in origin, i.e. they are controlled by many genes and genic interactions that cannot be defined. Thus, inferences of genetic structure are necessarily obtained indirectly from studies of phenotypic variation.

Relative to polygenic quantitative traits, the electrophoretic study of allozyme variants (alleles) of isozymes associated with specific gene loci allows direct estimates of genetic variation to be made within and among populations. These genetic markers are not commonly associated with observed phenotypic characters and are expected to be neutral in nature, i.e. they act as random variants that are not influenced by natural or directed selection. Furthermore, electrophoretic techniques allow different sets of questions to be posed regarding the amount, distribution, and maintenance of genetic variation in both natural and artificial populations. When applied to natural or artificial breeding populations, this technique gives the breeder the opportunity to certify orchards, families, and clone banks, as well as being able to monitor breeding processes in seed production areas, seed collection areas, and/or seed orchards. An added benefit is that results can often be obtained in months, not years.

There is a great diversity of tissues that can be used for electrophoretic analysis. However, the most appropriate type of tissue is often directly related to the species, and the questions being posed. Thus, it is extremely important that the questions be clearly formulated prior to sampling.

1.1 BRIEF OVERVIEW OF ELECTROPHORESIS

Electrophoresis is a process whereby proteins, introduced into a molecular sieving media, are forced to migrate through this sieve under the influence of an electric current. The total distance that a protein migrates depends upon the pH of the buffer systems, the net electric charge of the protein, and its three dimensional structure.

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The molecular sieve can be composed of many different substances. Although these can include paper, agar, acrylamide, and starch, we will consider only horizontal starch-gel electrophoresis throughout this manual. Regardless of the media, however, the basic procedures and principles remain the same.

Starch gels afford a researcher several immediate advantages over other types of media for population-type surveys. Probably the most significant is the ability to ascertain the genotype of an individual for a larger number of gene loci in one run. This allows for rapid construction of the genetic composition of a population, and/or the multilocus identity of an individual.

For routine population genetics surveys, proteins are absorbed onto paper wicks from a crude homogenate prepared from the selected tissue. Usually, preparation of the crude homogenate simply involves crushing the selected tissue in an appropriate extraction buffer. After the wicks are inserted into gels, electric power is applied to the system and migration of the buffer-front is tracked for about 6-8 cm (ca. 4-5 hours of electrophoresis). The gels are then sliced horizontally into approximately 1-2 mm thick slices and are used to visualize specific enzymatic proteins. After the zones of enzyme activity are developed, they are characterized and scored for the different enzyme systems being studied.

In addition to providing general laboratory procedures for starch gel electrophoresis, we discuss types of tissues that can be utilized and some of the data that can be obtained from these tissues. Specific laboratory procedures are indicated for different types of tissues for both conifers and angiosperms.

2 TISSUES FOR ANALYSIS

Many different types of tissue can be collected for an electrophoretic investigation. The type of tissue collected dictates not only the laboratory procedure employed, but the type of data that can be collected. Because of their reproductive biology, conifers offer variety in the types of data that can be collected. However, the researcher must know exactly what data are required in order to collect them in the most efficient way.

Mature needles from coniferous species are probably the most difficult to analyse electrophoretically. In general, mature vegetative tissues from perennials are rich in tannins, quinones, phenols, flavonoids, etc. These compounds will bind to active sites of most enzymes, and have made large-scale investigations using many enzymes difficult. However, several procedures have been developed in our laboratory which allow large-scale experiments to be conducted using many enzyme systems (Pitel and Cheliak 1984). We can analyse numerous genotypes with relative ease.

We will first describe the varieties of tissue that can be analysed, the procedures employed, and the types of data that can be obtained.

2.01 Types of tissue

2.011 Seeds

In general, seeds of forest trees are attractive for electrophoretic surveys of genetic variation. Some advantages and disadvantages of utilizing seed are:

Advantages

1. Storage conditions are well known for most species.

2. Small space is required to store large numbers of genotypes.

3. Optimal storage conditions are usually easily satisfied.

4. Tissues are relatively "clean" (i.e. free of tannins, phenols, etc.).

5. In conifers, inheritance and linkage studies can be made directly without doing crosses.

Disadvantages

1. Seed crop periodicity may severely restrict the size and type of sample that can be collected.

2. Only sexually mature individuals can be sampled.

3. Low germinability and/or special requirements for overcoming dormancy impose limitations of sampling and resources for some species.

4. In angiosperms, only embryos are suitable for a survey of genic variation in the populations. However, this filial generation may not be able to provide data appropriate to answer the questions being asked.

5. In conifers, the use of haploid gametophytic tissue to derive the genotypic structure of a population is the most inefficient, time consuming, and expensive way to collect the required data.

6. Except for serotinous-coned conifers, seed collections have to be made in a relatively short period of time in the late spring or early fall. This timing constraint can present logistical problems in organizing and successfully executing large-scale collections of a species.

2.012 Vegetative tissues

For our purposes, we define vegetative tissues as all tissues directly representing the genotype of the individual from which it was collected. Thus, we can consider roots, leaves, buds, cortical tissue, etc., as vegetative tissues.

Advantages

1. Diploid genotype is directly expressed for both gymnosperms and angiosperms

2. Plants can be sampled at any stage of development from seedling to maturity

3. Most tissues are available at most times of the year

4. Sample can include as many individuals within a population as required

Disadvantages

1. Long-term laboratory storage conditions are unknown for most species

2. Storage space can be extensive relative to a comparable sample of genotypes for seeds

3. Storage may require specialized conditions (i.e. a biological ultra cold freezer)

4. Tissues are often quite "dirty" (i.e. rich in phenols, etc.)

5. Inhertiance and linkage have to be confirmed by pedigreed material

2.1 SEXUAL REPRODUCTION IN CONIFERS

The sexual reproductive process in conifers can be both a benefit and a problem for electrophoretic procedures if its implications are not fully understood. In the female reproductive structure, meiosis results in a linear tetrad of four gametes. The three non-polar gametes degenerate, leaving one haploid cell. This cell undergoes vegetative reproduction (mitosis) to produce a large mass of undifferentiated cells. Depending upon the genera, this process can take up to 13 months to complete. Most cells eventually differentiate to form a nutritive tissue (endosperm = megagametophyte) and several archigonia. The important point is that this mass of tissue has originated from a single haploid cell and remains in this condition after fertilization. The mature embryo is diploid and has inherited the same haploid genotype as the megagametophyte, with the addition of the paternal haploid contribution from pollen upon fertilization (Figure 1).

2.12 Sampling procedures for various types of information

This reproductive system allows many different types of information to be extracted. Therefore, it is extremely important that the researcher know exactly what type of information is needed prior to starting a study based on haploid tissue from sexually mature conifers.

2.121 Inheritance and linkage

Given the reproductive system of conifers, we can perform inheritance and linkage studies without physically doing the crosses. If a tree is heterozygous at a particular gene locus, half of the gametes (megagametophytes) will include one allele (say A) and the remainder the alternate allele (say a), if the gene locus behaves in a simple Mendelian fashion. Thus, we can form a null hypothesis to test that, in a heterozygous tree, the segregation of alleles (A,a) is 1:1 at a single locus. Because analysis of a megagametophyte is conditional on pollination and fertilization, this hypothesis necessarily

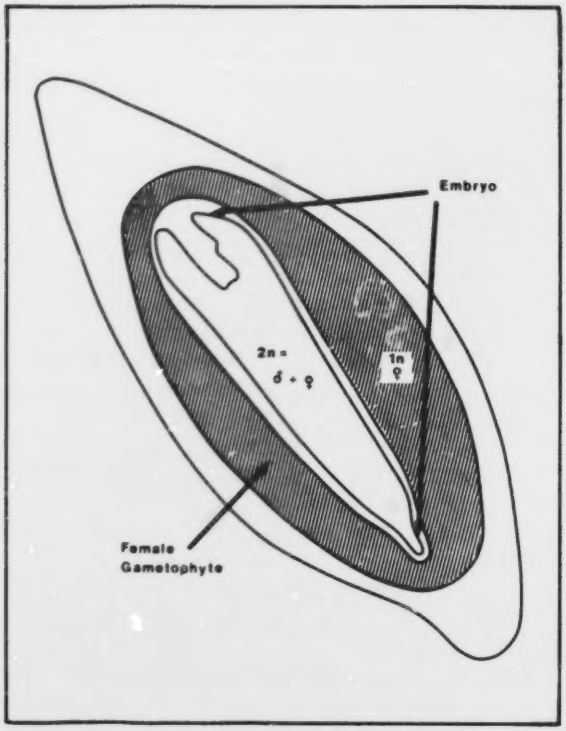

Figure 1. Genotypic composition of a mature conifer seed.

assumes no segregation disortion, that segregation is being observed at meiosis, and that the separate embryos, including maternal alleles '$\underline{A}$' and '$\underline{a}$', have equal fitness.

To test for linkage, suppose that an individual is heterozygous for two alleles at two loci say $\underline{Aa}$ and $\underline{Bb}$. If these two loci are not linked, a 1:1:1:1 distribution of the gametes, $\underline{AB}$ $\underline{Ab}$ $\underline{aB}$ $\underline{ab}$, should be observed. In this way we can test all $[n(n-1)]/2$ pairs of heterozygous loci in an individual for possible linkage using a χ^2 or maximum likelihood G or G^2 analysis.

2.122 Genotype distributions

We can predict the genotype of an individual by using haploid cells. The probability of identifying a heterozygote correctly, given that it is a heterozygote, follows the density function $P_h = 1-[(1/2)^{n-1}]$, where n is the number of megagametophytes and P_h is the probability of observing a heterozygote. By analysing eight megagametophytes, the probability of observing a heterozygote is >0.996. However, these data simply represent the diploid constitution of an individual. Thus, using single megagametophytes takes 8-10 times as much work, time, and expense to derive the diploid genotype of an individual compared to direct observation from somatic tissues. If the required data are the individual gentoypes, it is better to bulk a number

(not <8) of megagametophytes, crush them simultaneously and then determine directly the diploid genotype. Generally, when working with conifers, an inheritance and linkage analysis will be conducted before the major study is undertaken. From this study, the diploid gel phenotypes from bulked megagametophytes can be constructed from known heterozyotes to allow easy interpretation of unknown individuals.

2.123 Gene frequency distributions

The haploid megagametophyte may also be used to determine gene frequency distributions from bulked samples of seed from a number of parents. The procedure is to simply count the number of different alleles observed in a bulked sample and divide by sample size.

For most polymorphic loci within a population, a bulked sample from 30-50 individuals (all contributing approximately equal seed) will suffice. Subsequent analysis of approximtely 2N-2.5N (N is the number of contributing individuals) megagametophytes will provide a reasonable estimate of the frequency of alleles in that population. Because most investigators reduce genotypic data sets into gene frequency, this method will allow accurate estimates of gene frequency to be obtained quickly and inexpensively. If an approximate genotypic distribution is required, it can be obtained from the Hardy-Weinberg equilibrium (i.e. $p^2:2pq:q^2$, where $p+q=1$) if we assume random mating.

2.124 Preparation of the samples

Seed should be prepared by standard techniques required to overcome dormancy in those species requiring pretreatment prior to germination. The treated seed is placed on filter paper in a plastic petri dish and soaked in water at room temperature for a minimum of 24 hours. Excess water is then poured off and the samples placed in refrigerator at 4°C. However, it is advisable to do some preliminary experiments by allowing seeds to germinate for varying lengths of time in order to determine the stage of maximum enzyme activity.

The seed coats of the treated seed are removed and the embryos excised in preparation for enzyme extraction. We find this tedious task easiest to accomplish with a good pair of sharp-tipped surgical forceps. Most species have two halves to the seed coat and pressure down the tension zone will split the exine and expose the megagametophyte. An incision is made in the top of the naked seed to allow the embryo to be pushed through the incision with gentle pressure from the bottom. Depending upon the experiment, the embryos may be discarded or retained.

The megagametophytes are placed singly in 0.5 ml auto analyse cups. One drop of seed extraction buffer (Appendix I) is added to each cup. Cups are covered with Saran wrap, or tightly fitting plastic caps and placed in the refrigerator in preparation for homogenization the following morning.

If the embryos are to be genotyped as well, we usually add about 15% of vegetative extraction buffer I (Appendix I) to the seed extraction buffer. These proportions can be adjusted depending upon the age of the embryos being studied.

2.2 SEXUAL REPRODUCTION IN ANGIOSPERMS

The endosperm in angiosperms is triploid (2n , 1n) with a diploid embryo. Therefore, the endosperm does not provide a suitable tissue to directly genotype an individual, and embryos must be used to characterize the population. However, there can be serious errors in this method if no account is taken of the mating system.

With vegetative tissue samples we are dealing directly with the diploid genotype of an individual. This can, and does, present problems when interpreting banding patterns in terms of their genetic relevance. Without physically doing confirmatory crosses or having access to pedigreed material, all we can do is make reasonable inferences of gene loci.

A study dealing with large populations and a number of gene loci can be conducted very quickly using diploid material. In our laboratory we can genotype up to 200 individuals per day, scoring each for up to 33 different enzyme systems. Thus, in 20 lab-days, a considerable amount of data can be collected with relatively little cost.

2.3 TYPES OF VEGETATIVE MATERIAL

Most of the living tissue of a mature plant can be used for an electrophoretic survey of genetic variation. Several points, however, should be kept in mind when planning the collection of the initial survey. Ease of collecting and storing the tissue must be a primary concern. Seeds are relatively easy to store. However, owing to seed crop periodicity, or if there is interest in knowing the structure of the mature population, it may be necessary to collect directly from the mature plant rather than sampling the filial population. Direct sampling of the standing crop may be necessary if the population to be investigated is not sexually mature. We will describe various types of tissue that can be sampled and an appropriate homogenization procedure for each.

2.31 Buds

We prefer buds to any other type of 'dirty' vegetative material. In temperate areas, buds are available in a dormant condition for 6-8 months of the year. With this tissue it is easy to standardize a sampling position and bud type (vegetative or reproductive). Storage conditions can usually be met by freezing in a chest freezer for at least one year. We have been successful in extracting enzymes from vegetative buds of most decidious species as well as conifers. Buds have an additional advantage in that they are not as rich in phenols and denaturing secondary metabolites as are most other types of mature vegetative tissue. Mechanization of the homogenization process is quite straightforward. Normally 10-50 mg of tissue is more than sufficient to provide a crude homogenate for 4-5 wicks.

2.32 Mature leaves

2.321 Angiosperms

Long term storage of mature leaves can be a problem. We would recommend using the tissue as fresh as possible, certainly within one week of collection. We have not been successful in freezing these tissues in whole or block form and still maintaining enzyme activity. The following procedures have been

in our laboratory: (1) quick freeze in liquid nitrogen for 3-10 seconds, with subsequent storage in a chest freezer (ca. -20°C); (2) slow freeze of tissue in a chest freezer. The results were the same, complete loss of enzyme activity except for several ubiquitous peroxidase loci. We recommend keeping the leaves cool (2-4°C), moist (80%RH), and using them within one week.

Although no microscope work was performed, we think the problem with freezing in block form is that the cell walls rupture, exposing enzymes to denaturing secondary metabolites. If long-term storage is necessary, we recommend that the tissues be homogenized and either frozen immediately or after centrifuging. Many other laboratories working with insects, mammalian blood, and annual plants have found this an effective method of storage for periods of years. Mechanization of the process can be accomplished with a little additional preparation.

2.322 Gymnosperms

Long term storage of mature leaves from gymnosperms can be accomplished using the procedure described for buds. We have stored dormant mature needle tissues in a regular chest freezer (ca. -20°C) for periods up to one year with no apparent decrease in enzyme activity.

For some species of conifers, (e.g. Douglas-fir Pseudotsuga menziesii [Mirb.] Franco and tamarack Larix laricina [DuRoi] K. Koch) an apparent ontogenetic effect is associated with the age of the needle tissue. First year needles do not contain sufficient amounts of active protein to be detected with the visualization methods used in electrophoresis. The reason for this is not well understood. Thus, second year foliage from the species should be tested as well as younger tissues. Also, we have had little success working with newly expanding needles, primarily from Picea. The problem may be that levels of phenolases and proteases are too high in these tissues to be inactivated with our extraction protocols. Therefore, we recommend that these tissues not be sampled until they have gone dormant for the first time.

As needle tissues are tough and have relatively low concentrations of protein, it is necessary to use a mortar and pestle to prepare the crude homogenate. After grinding, the homogenate is placed in auto analyse cups for storage. If storage is required overnight, homogenates can be placed in a refrigerator (ca. 4°C), with a Saran wrap covering, or tightly fitting plastic caps, to prevent dessication. However, for longer term storage, we recommend covering the cups and placing them in a freezer at about -20°C.

These needle tissues present some new problems for extraction buffers. Details are reported elsewhere (Pitel and Cheliak 1983). We have had good success by using about 75-100 mg of tissue with about 0.5 ml of vegetative extraction buffer II (Appendix I).

2.33 Roots, bark, and cambial tissues

Actively growing root tips, especially from young seedlings, are a source of tissue from which enzymes can be extracted easily. Mechanization is straightforward as the tissues are soft and succulent. Because the tissue is reasonably clean, a modified version of vegetative extraction buffer I (decreasing the amounts of PVP 360 M) can be used successfully. In general

5-30 mg of fresh tissue with 0.1-0.5 ml of extraction buffer is sufficient for 3-5 wicks.

In some instances it may be necessary, or desirable, to use bark and cambial tissues. Because this tissue is tough and has low concentrations of protein, homogenization is best accomplished with a mortar and pestle. We have had success extracting enzymes from this tissue by using vegetative buffer I. In a number of species, the number of enzymes obtained from bark is typically less than that for buds or needles. Thus, it is recommended that bark and cambial tissue be used only if alternatives are unavailable.

Samples of bark tissue were obtained during the dormant period of the year. Larger diameter branches (ca. 5-10 mm) were clipped and placed in heavy plastic bags and stored at -20°C until analysis. Usually 200 mg of bark was stripped from the branch and placed in 0.5 ml of extraction buffer in a cooled mortar for grinding.

3 PREPARATION OF THE CRUDE HOMOGENATE

The objective of homogenization is to rupture cells of the tissue and introduce enzymes into a buffered extraction solution. A large number of methods are available from the literature for homogenization. However, the majority of these methods require manual grinding of a relatively large amount of tissue. Where possible, we have mechanized the homogenization procedure. This decreases the time spent preparing samples. Tissues which are not amenable to mechanized homogenization include conifer needles, mature leaves of some angiosperms, and bark and cambial tissues of all species. In these cases, the amount of tissue required is reduced to a minimum.

3.1 MATERIALS

3.11 Mechanized homogenization

Routinely, 0.5 ml auto analyse cups with conical bottoms were used as grinding vessels. They are inexpensive (ca. 0.1-0.3 cents each), durable and, most importantly, strictly for one-time use and disposable. This eliminates contamination between samples. A peg-board, with appropriate size holes, is used to hold the sample cups. These inexpensive boards can be made in a number of different ways to suit the sampling strategy of a particular study.

The grinding pestle consists of a teflon head, which fits the inside contour of the sample cup, attached to 1/4" drill rod. This assembly is then fitted into a motorized grinder, preferably of low rpm and high torque. (Figure 2).

Grinding megagametophytes or small embryos normally can be accomplished in less than five seconds. For tougher vegetative tissues a knurled teflon grinding head is helpful, but not essential. This tissue usually can be homogenized in less than 15 seconds per sample. It is important to keep the

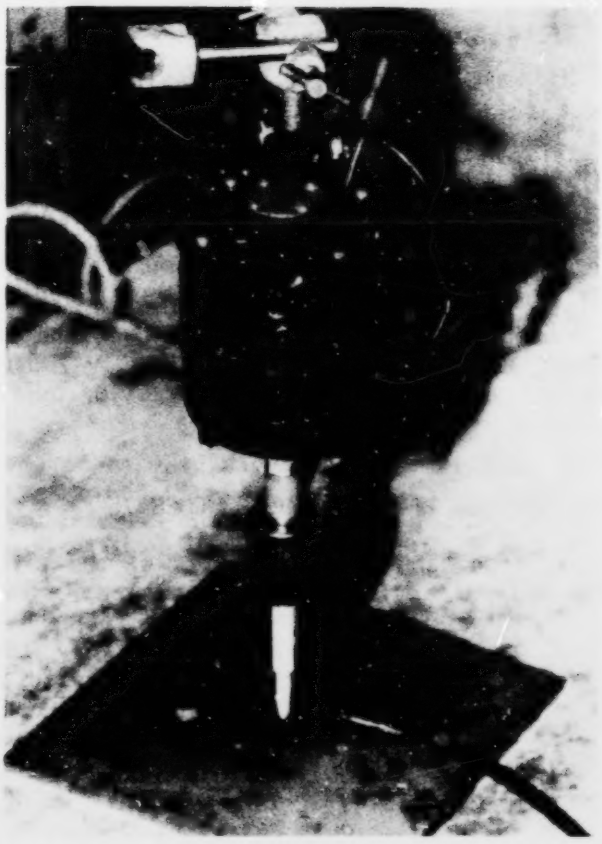

Figure 2. Mechanized vegetative tissue and seed homogenizer.

homogenate cool during the entire grinding process because many proteins can be denatured by heating. They are, however, not adversely affected by the cold. For example, if egg albumin (a protein) is frozen, it can be thawed and will return to the pre-freezing state. However, if the egg is fried it cannot be returned to its previous state. Essentially, the same occurs to some enzymatic proteins upon heating. Therefore, it is essential to keep all operations, except for incubation, below about 4°C.

3.12 Hand grinding

For tough vegetative tissues, we use a small mortar and pestle with sand. To minimize grinding time, as well as the possibility of overloading protein in the gel, the smallest amount of tissue from which the enzymes can be visualized is homogenized. Usually, 50-200 mg of tissue with 0.5-1.5 ml of extraction buffer is sufficient. In general, the maximum amount of extraction buffer should be added to a minimum amount of tissue. This will assure us that the enzymes are extracted without being denatured by secondary metabolites in tissues. Generally, it will be necessary to determine the optimal tissue/extraction buffer ratios for different species and laboratories by trial and error.

4 SAMPLE WICKS

After homogenization, crude extracts have to be absorbed onto material suitable for introduction into the gels. Absolute size and weight of the wicks will depend upon the specific gel set-up and concentrations of proteins in the homogenate. Width of the sample wicks also affects the number of positions that can be loaded into a gel as well as the number of wicks that can be obtained from a given sample.

4.1 SIZE

To utilize the depth of the gel to best advantage, the wicks should be slightly longer than the final gel depth. The width of the wick can affect the resolution of a protein. This is particularly evident at higher power (electric potential). In general, as the power applied to the gel increases the width of the sample wicks should also increase. However, for most routine work, whether diploid or haploid tissues, we use a 1.5 mm wide wick. This width allows us to load between 60-70 positions within the gels and provides good resolution. Wicks 2-4 mm in width are recommended to photograph gels for illustrative purposes.

4.2 WEIGHT

The weight of the wick affects not only the number that can be obtained from a particular sample but also the concentration of the protein loaded into the gel. Poor resolution will result if too much protein, in absolute amounts, is loaded into the gel. Thus, it may be necessary to experiment with various wick weights for subsets of enzymes scored in a given gel. Additionally, the ratio of tissue/extraction buffer can be adjusted to help in resolving these problems. We usually use sample wicks made from No. 3 Whatman filter paper. However, if a suitable compromise cannot be obtained by adjusting the weight of the wicks and concentration of a particular protein, it may be necessary to reduce the amounts of substrates and co-factors added to a staining recipe.

4.3 INSERTION INTO THE HOMOGENATE

The intended number of wicks to be run on a particular day should be inserted simultaneously into the homogenate. This will ensure that all wicks receive approximately equal amounts of the homogenate. We recommend that wicks be inserted into the homogenate just prior to loading of the gels, thereby reducing the amount of homogenate lost through evaporation.

5 STARCH GEL PREPARATION AND ELECTROPHORESIS

5.1 GEL MOLDS AND BUFFER TANKS

There are many arrangements available in which the gels can be cast. The following system is quick, flexible, and relatively inexpensive. The set-mold that we use is modified slightly from that of F.C.H. Yeh (B.C. Min. Forests). Molds for the gels are formed by four plexiglass strips (26 mm x 260 mm or 177 mm x 12 mm), two of each size, secured to a plexiglass plate 177 mm x 260 mm x 12 mm with large paper clamps on the 177 mm sides to hold the ends

secure and small paper clamps on the 260 mm side to serve as a guides (Figure 3). After assembly, the whole arrangement is leveled with a small spirit level. This is important because variation in thickness of the gel results in variable power and, hence, variable migration rates of the buffer front and proteins.

Buffer tanks are made from "Rubbermaid" drawer organizers (7 cm x 23 cm). A hole is drilled in the top of one end and fitted with a colour coded female banana plug onto which a platinum wire is silver soldered. Tanks are then placed in a wooden form to prevent accidental spills (Figure 4). Disposable absorbant cloths (J-Cloths) are used as bridge wicks to complete the circut between electrolyte solutions in the buffer tanks and gel.

5.2 GEL PREPARATION

Gels are prepared as the first step each morning to allow sufficient time for cooling to about 4°C. Although gels prepared the previous day and kept cool overnight seem to have added strength, resolution of proteins seems to be reduced dramatically. Therefore, we do not recommend this procedure.

The appropriate amount of starch is weighed out the previous night and placed in a thick-walled 1000 ml Erlenmeyer flask with a side evacuation sleeve. Buffer concentrates are decanted, diluted to the correct volumes, and placed in the refrigerator. Both flasks are covered with parafilm.

Approximately 1/5 of the buffer is added to the dry starch to make a suspended solution, free of lumps. The remaining buffer is heated, either in a microwave oven or with a bunsen burner, until it is rapidly boiling. Approximately 1/2 of this heated buffer is added initially to the starch suspension and swirled vigorously. The starch suspension will become quite viscous. The remaining buffer is quickly added, and again swirled vigorously. This starch solution is then returned to the heat until it begins to boil throughout. The starch is "cooked" when the solution becomes semi-transparent, in contrast to opaque. At this point the cooking procedure is stopped.

It is necessary to apply a vacuum to the solution until only large bubbles are left (usually 20 seconds to 2 minutes depending upon the buffer solution). The solution is then poured into the gel molds and allowed to set. After about 20 minutes a plastic film covering should be placed on the gel to prevent dehydration. The molds are placed in the refrigerator. The gels are ready for use after they have been trimmed of their excess starch and have cooled to about 4°C.

5.3 APPLICATION OF SAMPLES TO THE GEL

To set the origin for placement of wicks, a cut is made through the gel approximately 2.5 cm from the intended cathodal end. This strip is pulled back about 5 mm to facilitate loading. A sample wick is carefully removed from a sample cup, dried lightly on a paper towel to remove any excess homogenate, and then placed against the anodal side of the cut in the gel. Care must be taken to guard against placing wicks too close together in the gel. Spacing should be maintained at 1-1.5 mm between wicks. We use red food coloring as a tracker dye and load it into the two slots on either end of the

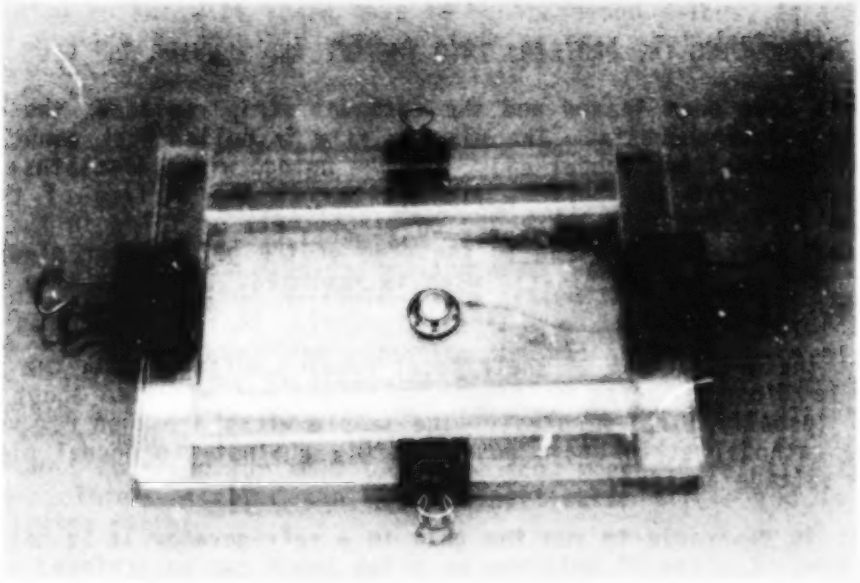

Figure 3. The gel mold.

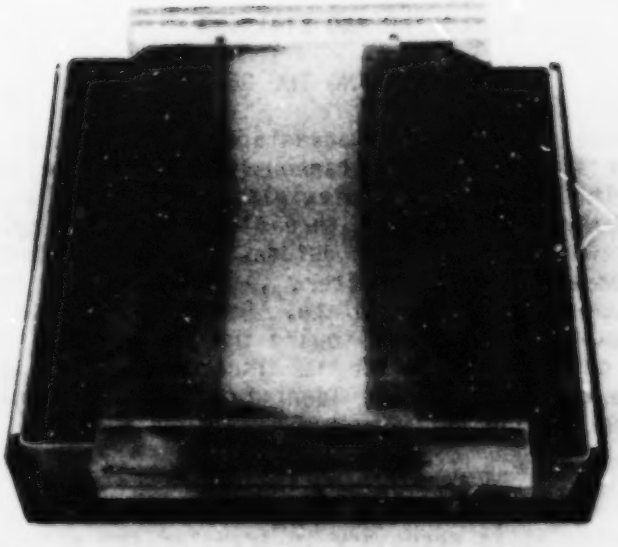

Figure 4. Gel mold and electrode buffer tank.

gel. After the gels have been loaded, the cathodal strip is pushed firmly back into its original position. It is extremely important that these two pieces of gel make firm contact with each other; otherwise, the buffer will not traverse cleanly into the starch slab.

5.4 INTRODUCING THE PROTEINS INTO THE GEL AND RUNNING THE GELS

After the gels have been loaded and the cathodal strip replaced, the samples are ready for electrophoresis. The plastic film is pulled back about 1.5 cm from both the cathodal and anodal ends, and the bridge wicks (J-cloths), which must be saturated with the buffer, are applied to the surface of the gel. Care is taken to ensure that there are no air pockets or folds in the bridge wicks, and that they fully extend to the edge of the gel. After placing the bridge wicks on the gel, the plastic wrap is replaced. Power is then applied to the system.

We start the gels at 0.5 of their total running voltage until the tracker dye (either red food coloring or bromphenol blue) has migrated about 3-5 mm into the gel (usually 10-15 minutes). The sample wicks are then removed from the gel, the cathodal strip again pushed firmly against the anodal slab, and full running voltage applied.

While it is desirable to run the gels in a refrigerator it is not absolutely necessary. Cooling of gels run on a lab bench can be achieved by placing crushed ice in a zip-lock bag or freezer packs in trays (filled with water) on a sheet of plexiglass on the upper surface of the gel.

Electrophoresis is continued until the buffer front (marked by the tracker dye) has migrated a sufficient distance. For the majority of species and tissues, 5-8 cm migration (3-5 hours of electrophoresis) is sufficient to clearly resolve and separate most stepwise charge variants.

6 SLICING AND STAINING

6.1 SLICING THE GELS

Compared to acrylamide gels a great advantage of starch gels is the opportunity to slice and stain them simultaneously for a large number of enzyme systems. To slice the gels, we use plexiglass guides (20 x 260 x 1 mm) and either nylon sewing thread (invisible thread) or low test nylon fishing line held tense while drawing it over the guides. A weight, usually a staining tray partially filled with ice cold water, is placed on the top of the gel to prevent it from slipping on the base plate. Guides are placed next to the gel and the line is drawn through the gel toward the person slicing. After reaching the top of the gel, the top slice is discarded and the gel is marked in some way to indicate one edge and its identity. We usually make a cut in the upper left-hand corner of the gel.

6.2 STAINING THE GELS

After cutting the gels, it is necessary to transfer the slices to the individual staining trays. This requires practice to avoid breaking the slices during transfer. Details of stain recipes can be found in Appendix II.

Because stains are expensive, we have reduced to a minimum the amounts of chemicals needed to stain several slices of gel. However, the thicker the gel slices, the more staining buffer required. Additionally, if the trays for staining are very large in relation to the size of the gel slice, the amount of staining buffer will again have to be increased. Thus, it is best to use as thin a slice as possible with a staining tray that just fits the size of the gel slabs being stained.

7 SCORING ALLOZYME VARIANTS

It is necessary to characterize allozyme variants to be able to record and score them consistently. There are several ways in which this can be done. The convention in most studies dealing with forest tree species has been to number isozyme loci (for multiple locus systems) sequentially from the anode to the cathode. Variant allozymes are then characterized according to their locus. This usually involves comparing the migration distance of the variant to some standard. Specifically, the migration distance of some common allozyme within a locus is measured and compared to the total distance that the buffer front has migrated. This is called the R_f of a particular locus. Any variants observed for that locus are then measured relative to the common allozyme. This is called the R_m of a particular allele and is obtained by the following ratio;

$$R_m = \frac{\text{migration distance of variant (mm)}}{\text{migration distance of standard (mm)}}$$

We assign a sequential numeric value to the new allozyme variant and associate that number with a particular R_m.

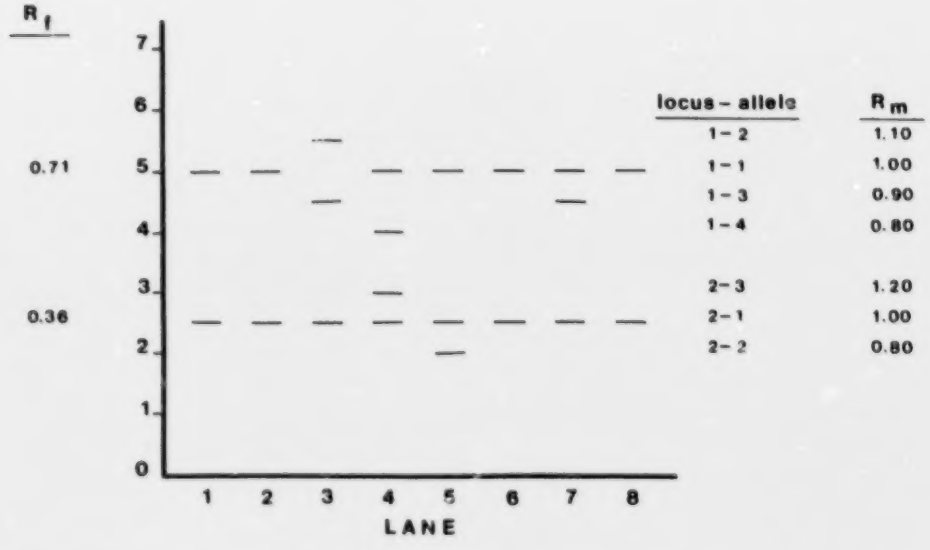

Figure 5. Hypothetical gel showing allozyme variants at two loci.

For example, consider the hypothetical gel indicated in Figure 5. There are two isozyme loci for this enzyme. For the first locus, the common allozyme migrates to R_f = 0.71; the common allozyme for the second locus migrates to R_f = 0.36. Within locus 1, four alleles have been detected. The common allele obviously has an R_m = 1.00. The additional allozymes at this locus (2, 3, 4) are all characterized by their distance of migration relative to allele 1. The same procedure is used to characterize the variants at locus 2.

Characterizing the isozymes and allozymes in this fashion within a species allows comparisons to be made with other laboratories, as long as electrophoretic conditions (buffer systems etc.) are constant. Migration distances and R_ms are only intended as a guide, not an absolute value. That is, they have a standard error associated with them. Slightly different R_ms will be obtained with different gel concentrations and small differences in pH. Thus, an allele that migrates to 0.96 in one instance is not necessarily different than one that migrates to 0.95 on another occasion.

ACKNOWLEDGMENTS

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APPENDIX I

Extraction Buffers

Seed extraction buffer

10 ml	Tris-citrate buffer (pH 7.0)
0.4 mM	NAD
0.2 mM	NADP
0.001M	Ascorbic acid
0.001M	EDTA (disodium)
0.1%	Bovine Serum Albumin

Adjust pH to 6.8-7.5 with 0.1M Tris and add 5 drops of β-mercaptoethanol.

Vegetative extraction buffer I

8%	PVP (7:1 of 40M; 360M)
0.3M	Sucrose
0.5 mM	EDTA
1 mM	Dithiothreitol
1 mM	Ascorbic acid
0.1%	Bovine Serum Albumin
0.4 mM	NAD
0.3 mM	NADP
0.2 mM	Pyridoxal 5'-phosphate

Adjust pH to 6.7 with 1M Tris and add 0.66 ml β-mercaptoethanol for 100 ml of buffer.

Vegetative extraction buffer II

2%	Tergitol 15-S-9
2%	PEG (20M)
8%	PVP (7:1 40M; 360M)
50 mM	Ascorbic acid
0.4 mM	NAD
0.1%	Bovine Serum Albumin
0.2 mM	Pyridoxal 5'-phosphate
0.3 M	Sucrose
12 mM	Cysteine-HCℓ
0.66 ml	β-mercaptoethanol

Dissolved in 0.05M borate buffer (pH 7.1); final volume of 100 ml. Re-adjust pH at end.

APPENDIX II

Gel, Electrode and Staining Buffers and Enzyme Recipes

APPENDIX II

Gel, Electrode, and Staining Buffers, and Enzyme Recipes

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Abbreviations

NAD: β-nicotinamide adenine dinucleotide
NADH: β-nicotinamide adenine dinucleotide, reduced form
NADP: β-nicotinamide adenine dinucleotide phosphate
EDTA: ethylenediaminetetraacetic acid
PEG: polyethylene glycol
PVP: polyvinylpyrrolidone
Tris: Tris (hydroxymethyl) aminomethane
NBT: nitro blue tetrazolium
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMS: phenazine methosulfate
ADP: adenosine-5'-diphosphate
ATP: adenosine-5'-triphosphate

Table A2.1. Composition of gel and electrode buffers used for starch gel electrophoresis and electrophoretic conditions

Name	Buffer		
	Gel	Electrode	Current
B	0.03 M Tris 0.005 M citric acid (anhydrous) 1% electrode buffer pH 8.5 (10X concentrate solution)	0.06 M lithium hydroxide 0.3 M boric acid pH 8.1	50 mA
H	0.05 M histidine-HCℓ 1.40 mM EDTA (pH 7.0 with 1.0 M Tris) Dilute stock, 4:1	0.125 M Tris pH 7.0 (with 1 M citric acid)	50 mA

Stain buffer recipes

(1) 0.5 M malic acid:

(a) dissolve 67.2 g. of DL-malic acid in 800 ml of distilled water.
(b) adjust pH to 7.0 with 10N NaOH
(c) bring volume to 1 litre.

(2) 0.2 M acetate, pH 5.0:

sodium acetate	11.49 g
glacial acetic acid	3.6 ml
distilled water	to 1 litre

(3) 0.2 M Tris - HCℓ, pH 8.0:

Trizma base	24.22 g
water	750 ml
adjust pH with HCℓ	
distilled water	to 1 litre

(4) 0.05 M citrate -0.05M phosphate, pH 4.0:

Na_2HPO_4 (anhydrous)	3.55 g
citric acid (anhydrous)	5.25 g
distilled water	500 ml

(5) 0.2 M phosphate (pH 7.0 or 6.4):

(a) Na_2HPO_4 28.4 g
(sodium phosphate, dibasic, anhydrous)
distilled water to 1 litre

(b) adjust pH with 1M NaH_2PO_4
(138 g per litre, of sodium phosphate, monobasic, monohydrate)

Stain chemicals kept as solutions

Reagent	Concentration	Storage
NAD	10 mg/ml	4°C
NADP	10 mg/ml	4°C
NBT	10 mg/ml	dark at 4°C
MTT	10 mg/ml(H)	dark at 4°C
PMS	5 mg/ml	dark at 4°C
$MgCl_2$	10 mg/ml (1%)	R.T.
$MgCl_2$	100 mg/ml (10%)	R.T.
glucose-1,6-diphosphate	0.1 mg/ml	4°C
malic acid, pH 7.0	0.5 M	R.T.
cis-aconitic acid, pH 8.0	5% (w/v)	4°C
isocitrate dehydrogenase	20 units/ml	4°C
glucose-6-phosphate dehydrogenase	25 units/ml	4°C
glucose-6-phosphate dehydrogenase	5 units/ml	4°C
hexokinase	250 units/ml	4°C
glyceraldehyde-3-phosphate dehydrogenase	150 units/ml	4°C
malate dehydrogenase	300 units/ml	4°C
aldolase	50 units/ml	4°C

Gel fixative solution:

400 ml	methanol
100 ml	acetic acid
1000 ml	water

Note:

R.T.: room temperature
H: gentle heating needed to dissolve

ENZYME RECIPES

Acid phosphatase (ACP; E.C.3.1.3.2)

Stain Recipe:

25 ml	0.2M sodium acetate buffer, pH 5.0
75 mg	sodium-α-naphthyl acid phosphate
25 mg	Fast Garnet GBC salt
2.5 ml	1% $MgCl_2$ (w/v)

Incubate in the dark until brown-black bands appear (about 90 min.).

Electrophoresis Buffer: H

Aconitase (ACO; E.C.4.2.1.3)

Stain Recipe

25 ml	0.2M Tris-HCl, pH 8.0
2.5 ml	5% cis-aconitic acid, pH 8.0 (w/v)
20 units	isocitrate dehydrogenase
0.5 ml	1% $MgCl_2$ (w/v)
0.5 ml	NADP
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until blue bands appear.

Electrophoresis Buffer: H

Adenylate kinase (AK; E.C.2.7.4.3)

Stain Recipe:

25 ml	0.2M Tris-HCl, pH 8.0
50 mg	glucose
25 mg	ADP (adenosine 5'-diphosphate)
125 units	hexokinase
25 units	glucose-6-phosphate dehydrogenase
0.5 ml	1% $MgCl_2$ (w/v)
0.5 ml	NADP
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 120 min.).

Electrophoresis Buffer: H

Alcohol dehydrogenase (ADH; E.C.1.1.1.1)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
5 ml	ethanol (95% v/v)
0.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 120 min.).

Electrophoresis Buffer: B

Aldolase (ALD; E.C.4.1.2.13)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
125 mg	fructose-1,6-diphosphate (tetrasodium salt)
38 mg	arsenic acid (disodium salt)
150 units	glyceraldehyde-3-phosphate dehydrogenase
0.5 ml	NAD
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 60 min.).

Electrophoresis Buffer: H

Alkaline phosphatase (ALP; E.C.3.1.3.1)

Stain Recipe:

50 ml	distilled water
25 mg	sodium β-naphthyl acid phosphate
25 mg	fast blue RR
60 mg	$MgSO_4.7H_2O$
1.0 ml	1% $MgCℓ_2$ (w/v)

Incubate in the dark at 37°C until blue bands appear.

Electrophoresis Buffer: H

Aspartate aminotransferase (AAT; E.C.2.6.1.1)

Stain Recipe:

2 mg	pyridoxal-5'-phosphate
50 mg	fast blue BB salt
25 ml	substrate solution.*

*Substrate solution:

5.30 g	L-aspartic acid and
0.70 g	α-ketoglutaric acid, dissolved in 1 litre of 0.2M Tris-HCℓ, pH 8.0. To dissolve, re-adjust pH to 8.0 with NaOH.

Electrophoresis Buffer: B

Incubate in the dark at 37°C until blue bands appear on a pink background (about 30 min.).

Electrophoresis Buffer: B

Catalase (CAT; E.C.1.11.1.6)

Stain Recipe:

(a) Soak gel in 50 ml of electrode buffer B, pH 6.5, for 20 min. at close to 0°C.

(b) Discard solution and soak gel for 60 sec. in:

1 g	potassium iodide
50 ml	distilled water
1 ml	acetic acid

(c) Wash 3X with water and add to the gel:

50 ml	distilled water
0.5 ml	3% H_2O_2

Catalase activity appears as white bands, while peroxidase activity appears as blue bands.

Electrophoresis Buffer: B

Cytochrome oxidase (CTO; E.C.1.9.3.1)

Stain Recipe:

25 ml	0.1M phosphate buffer, pH 7.2
18 mg	α-naphthol
18 mg	N,N-dimethyl-p-phenylenediamine
18 mg	sodium azide

Incubate in the dark at 37°C until bands appear.

Electrophoresis Buffer: B

Diaphorase (DIA; E.C.1.6.4.3)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
0.5 mg	2,6-dichlorophenol-indophenol
13 mg	β-NADH
0.5 ml	MTT

Incubate in the dark at 37°C (about 45 min.).

Electrophoresis Buffer: B

Esterase (colorimetric) (EST; E.C.3.1.1.1)

Stain Recipe:

25 ml	0.2M phosphate buffer, pH 6.4
25 mg	each of α- and β-naphthyl acetate in 2.5 ml of acetone
50 mg	fast blue RR salt

Incubate in the dark at R.T. until bands appear (about 60 min.).

Electrophoresis Buffer: H,B

Esterase (fluorescent) (F-EST; E.C.3.1.1.1)

Stain Recipe:

10 ml	0.2M sodium acetate buffer, pH 5.0
1 mg	4-methylumbelliferyl acetate (in 3 ml of acetone)

Add dropwise to the gel. Read under a UV light at 366 nm. after a few minutes to about one hour, depending on the species.

Electrophoresis Buffer: H

Fumarase (FUM; E.C.4.2.1.2)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
200 mg	fumaric acid, disodium salt
300 units	malate dehydrogenase
2.0 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear.

Electrophoresis Buffer: H

β-galactosidase (β-GAL; 3.2.1.23)

Stain Recipe:

5 ml	0.05M citrate-phosphate buffer, pH 4.0
10 mg	4-methylumbelliferyl-β-D-galactoside (in 1 ml N,N-dimethyl formamide)

Incubate gels at 37°C for 30-45 min. View under UV light (366 nm.). Enzyme activity appears as bands of fluorescence. Photograph immediately through a yellow filter. Note: α-galactosidase can be stained by using the "α" form of the above substrate.

Electrophoresis Buffer: H

General protein

Stain recipe:

2 g	Amido Black
500 ml	methanol
500 ml	distilled water
100 ml	glacial acetic acid

Mix and filter before use. Stain gels for 30 min. at R.T. Destain with fixative solution.

β-glucosidase (β-GLU; E.C.3.2.1.21)

Stain Recipe:

5 ml	0.05M citrate-phosphate buffer, pH 4.0
10 mg	4-methylumbelliferyl-β-D-glucoside

Incubate gels for 45 min. at 37°C. View under UV light (366 nm.). Enzyme activity appears as bands of fluorescence. Photograph immediately through a yellow filter.

Electrophoresis Buffer: H

Glucose dehydrogenase (GLUDH; E.C.1.1.1.47)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
250 mg	glucose
1.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until bands appear.

Electrophoresis Buffer: H

Glucose-6-phosphate dehydrogenase (G6PD; E.C.1.1.1.49)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
100 mg	glucose-6-phosphate
0.5 ml	1% $MgCℓ_2$ (w/v)
0.5 ml	NADP
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 90 min.).

Electrophoresis Buffer: H

Glutamate dehydrogenase (GDH; E.C.1.4.1.3)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
200 mg	L-glutamic acid
0.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate the gels in the dark until dark blue bands appear (about 120 min.).

Electrophoresis Buffer: B

Glutamate-pyruvate-transaminase (GPT; E.C.2.6.1.2)

Stain Recipe:

5 ml	0.2M Tris-HCℓ, pH 8.0
20 mg	L-alanine
10 mg	α-ketoglutaric acid
150 units	lactate dehydrogenase
15 mg	NADH

Add dropwise to the gel. View under UV light (366 nm.). Enzyme activity appears as dark bands of defluorescence. Photograph immediately through a yellow filter.

Electrophoresis Buffer: H

Glyceraldehyde-3-phosphate dehydrogenase (G₃PDH; E.C.1.2.1.12)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
125 mg	fructose-1,6-diphosphate (tetrasodium salt)
38 mg	arsenic acid (disodium salt)
50 units	aldolase
0.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 120 min.).

Electrophoresis Buffer: H

Glycerate-2-dehydrogenase (G₂DH; E.C.1.1.1.29)

Stain Recipe:

5 ml	0.2M Tris-HCℓ, pH 8.0
20 ml	distilled water
50 mg	DL-glyceric acid (hemi-calcium salt)
1.0 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear.

Electrophoresis Buffer: H

α-glycerophosphate dehydrogenase (α-GPDH; E.C.1.1.1.8)

Stain Recipe:

(a) Substrate solution:

1.0M DL-α-glycerophosphate, disodium salt, pH 7.0

(b) Combine:

2.5 ml	above substrate solution
25 ml	0.2M Tris-HCℓ, pH 8.0
1.0 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear.

Electrophoresis Buffer: H

Hexokinase (HK; E.C.2.7.1.1)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
25 mg	glucose
25 units	glucose-6-phosphate dehydrogenase
6 mg	ATP
1 ml	1% $MgCℓ_2$ (w/v)
0.5 ml	NADP
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C.

Electrophoresis Buffer: H

Hexoseaminidase (HA; E.C.3.2.1.30)

Stain Buffer:

5 ml	0.05M citrate-phosphate, pH 4.0
10 mg	4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside

Add dropwise to the gel and incubate for 45 min. at 37°C. Rinse gel and sprinkle with 7.4N NH_4OH. View under UV light (366 nm.). Enzyme activity appears as bands of fluorescence.

Electrophoresis Buffer: H

Isocitrate dehydrogenase (IDH; E.C.1.1.1.42)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
100 mg	DL-Isocitric acid, trisodium salt
0.5 ml	1% $MgCℓ_2$ (w/v)
0.5 ml	NADP
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 60 min.). Note: IDH is usually stained on the same gel as PGM (check PGM recipe).

Electrophoresis Buffer: H

Leucine-amino peptidase (LAP; E.C.3.4.11.1)

Stain Recipe:

(a)	50 ml	0.5M boric acid
(b)	25 ml	0.2M Tris-HCℓ, pH 8.0
	25 ml	0.2M maleic anhydride

Mix and adjust pH to 5.3 with 1.0N NaOH just prior to staining.

(c)	0.5 ml	10% $MgCℓ_2$
	25 mg	L-leucine β-naphthylamide-HCℓ
	35 mg	fast black K salt

Soak gel in (a) for 15 min. Empty the solution. Mix (b) and (c) and add to the gel. Incubate in the dark at 37°C. Note: the need to soak the gel in (a) will depend on the species.

Electrophoresis Buffer: B

Malate dehydrogenase (MDH; E.C.1.1.1.37)

Stain Recipe:

12.5 ml	0.2M Tris-HCℓ, pH 8.0
12.5 ml	0.5M DL-malic acid, pH 7.0
0.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear (about 45 min.).

Electrophoresis Buffer: H

Malic enzyme (ME; E.C.1.1.1.40)

Stain Recipe:

12.5 ml	electrode buffer H
12.5 ml	0.5M DL-malic acid, pH 7.0
0.5 ml	1% $MgCl_2$ (w/v)
0.5 ml	NADP
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear.

Electrophoresis Buffer: H

Mannose-6-phosphate isomerase (MPI; E.C.5.3.1.8)

Stain Recipe:

25 ml	0.2M Tris-HCl, pH 8.0
25 mg	D-mannose-6-phosphate, barium salt
50 units	phosphoglucose isomerase
40 units	glucose-6-phosphate dehydrogenase
2.5 ml	1% $MgCl_2$
0.5 ml	NADP
0.5 ml	NBT
0.5 ml	PMS

Incubate in the dark until dark blue bands appear.

Electrophoresis Buffer: H

Menadione reductase (MR; E.C.1.6.99.2)

Stain Recipe:

75 ml	0.2M Tris-HCl, pH 7.0
25 mg	menadione
25 mg	NADH
1 ml	NBT

Incubate in the dark at 37°C.

Electrophoresis Buffer: H

Nicotinamide adenine dinucleotide dehydrogenase (NADHDH; E.C.1.6.99.3)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
12.5 mg	NADH
1 ml	NBT

Incubate in the dark at 37°C.

Electrophoresis Buffer: B

Nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPHDH; E.C.1.6.99.1)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
12.5 mg	NADPH
1 ml	NBT

Incubate in the dark at 37°C.

Electrophoresis Buffer: B

Peptidase (PEP; E.C.3.4.13.11)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
30 mg	L-leucyl-L-alanine
30 mg	L-leucyl-glycyl-glycine
30 mg	glycyl-L-leucine
5 mg	crude peroxidase
5 mg	snake venom (Crotalus viridis helleri)
25 mg	O-dianisidine, dihydrochloride
2.5 ml	1% $MgCℓ_2$ (w/v)

Incubate at R.T. for at least 6 hours until brown bands appear.

Electrophoresis Buffer: B

Peroxidase (PER; E.C.1.11.1.7)

Method #1

Stain Recipe:

(a) 15 ml 1% H_2O_2

(b) 15 ml 20% acetic acid (v/v)
300 mg benzidine, dihydrochloride
Mix together.

Mix (a) and (b) and add to the gel. Incubate at R.T. until blue bands appear. Photograph immediately.

Note: Peroxidase activity can also be obtained with the Catalase stain recipe. Extreme caution should be taken when using benzidine.

Electrophoresis Buffer: B

Method #2

Stain Recipe:

40 ml	0.2M Na acetate, pH 5.0
50 mg	3-amino-9-ethyl-carbazole, dissolved in 5 ml of N,N-dimethylformamide
2 ml	0.1M $CaCl_2$ solution
1 ml	3% hydrogen peroxide

Phosphoenolpyruvate carboxylase (PC; E.C.4.1.1.31)

Stain Recipe:

(a) 1.0 ml 1.0M Tris-HCl, pH 7.0
5.0 ml distilled water
250 mg PVP
0.5 ml 10% $MgCl_2$ (w/v)
50 mg phosphoenol pyruvate
10 mg sodium bicarbonate

(b) 50 mg fast blue BB salt in 2 ml of distilled water.

Pour (a) onto gel, and incubate at 37°C for 20 min. Pour (b) onto gel and mix into (a).
Incubate in the dark at 37°C.

Electrophoresis Buffer: B

6-phosphogluconate dehydrogenase (6PGD; E.C.1.1.1.44)

Stain Recipe:

5 ml	0.2M Tris-HCℓ, pH 8.0
10 mg	6-phosphogluconic acid, trisodium salt
1 ml	1% $MgCℓ_2$ (w/v)
1 ml	NADP
1 ml	MTT
0.5 ml	PMS

Add dropwise to the gel. Incubate in the dark at 37°C until dark blue bands appear (about 45 min.).

Electrophoresis Buffer: H

Phosphoglucose isomerase (PGI; E.C.5.3.1.9)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
12.5 mg	fructose-6-phosphate, disodium salt
5 units	glucose-6-phosphate dehydrogenase
0.5 ml	1% $MgCℓ_2$ (w/v)
0.5 ml	NADP
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C until dark blue bands appear.

Electrophoresis Buffer: B

Phosphoglucomutase (PGM; E.C.2.7.5.1)

Stain Recipe:

25 ml	0.2M Tris-HCℓ, pH 8.0
150 mg	glucose-1-phosphate, disodium salt
0.25 ml	glucose-1,6-diphosphate solution (0.1 mg/ml water)
25 units	glucose-6-phosphate dehydrogenase
0.5 ml	1% $MgCℓ_2$ (w/v)
0.5 ml	NADP
0.5 ml	MTT
0.5 ml	PMS

Incubate in the dark until dark blue bands appear (about 60 min.).
Note: Both PGM and IDH are usually stained on the same gel. To develop both enzymes, DL-isocitric acid (100 mg) and NBT (0.5 ml) are added to the above solution.

Electrophoresis Buffer: H

Polyphenol oxidase (PPO; E.C.1.14.18.1)

Stain Recipe:

25 ml	0.1M phosphate buffer, pH 7.2
18 mg	catechol
18 mg	N,N-dimethyl-p-phenylenediamine
18 mg	thiourea

Incubate in the dark at 37°C.

Electrophoresis Buffer: B

Pyruvate kinase (PK; E.C.2.7.1.40)

Stain Recipe:

5 ml	0.2M Tris-HCℓ, pH 8.0
25 mg	phosphoenolpyruvate, trisodium salt
2.5 mg	ADP
1 mg	fructose-1,6-diphosphate
1 mg	EDTA
25 mg	KCℓ
150 units	lactate dehydrogenase
15 mg	NADH
5 mg	$MgC\ell_2$

Add dropwise to the gel. View under UV light (375 nm). Enzyme activity appears as bands of defluorescence.

Shikimic acid dehydrogenase (SDH; E.C.1.1.1.25)

Stain Recipe:

50 ml	0.2M Tris-HCℓ, pH 8.0
25 mg	(-) shikimic acid
1.0 ml	1% $MgC\ell_2$ (w/v)
0.5 ml	NADP
1.0 ml	NBT
0.5 ml	PMS

Incubate in the dark at 37°C.

Electrophoresis Buffer: H

Succinate dehydrogenase (SUDH; E.C.1.3.99.1)

Stain Recipe:

25 ml	0.1M phosphate buffer, pH 7.0
75 mg	succinic acid, disodium salt
100 mg	EDTA, disodium salt
12.5 mg	ATP
0.5 ml	NAD
0.5 ml	NBT
1.0 ml	MTT
0.5 ml	PMS

Incubate in the dark at 37°C.

Electrophoresis Buffer: H

Superoxide dismutase (SOD; E.C.1.15.1.1)

Stain Recipe:

25 ml	0.05M Tris-HCℓ (pH 8.2)
1 mg	riboflavin
74 mg	EDTA, disodium salt
0.25 ml	NBT

Incubate gels in the staining solution at 37°C in the dark for 20 min. Place on fluorescent light box and illuminate for 20-30 min.

Electrophoresis Buffer: B, H

Additional Recipes:

Enzymes that were successful in other studies but were not successful for the five species in the vegetative experiment (Pitel and Cheliak 1984).

L-amino acid oxidase

Stain Recipe:

25 ml	0.14M Tris-HCℓ, pH 7.5
69 mg	L-leucine
9 mg	2,3,5-triphenyl tetrazolium chloride
0.5 ml	PMS

Choline dehydrogenase

Stain Buffer:

25 ml	0.05M Tris-HCℓ, pH 7.5
175 mg	choline chloride
25 mg	$MgSO_4$
1.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Galactose dehydrogenase

Stain Buffer:

25 ml	0.05M Tris-HCℓ, pH 7.5
225 mg	D(+) galactose
25 mg	$MgSO_4$
1.5 ml	NAD
0.5 ml	NBT
0.5 ml	PMS

Monoamine oxidase

Stain Recipe:

25 ml	0.1M phosphate, pH 7.2
30 mg	tryptamine-HCℓ
1.5 ml	NBT

Sucrose phosphorylase

Stain Recipe:

25 ml	0.003M phosphate, pH 6.9
1,700 mg	sucrose
25 mg	2,3,5-triphenyl tetrazolium chloride

Sorbitol dehydrogenase

Stain Buffer:

50 ml	0.05M Tris-HCl, pH 8.0
250 mg	D-sorbitol
1 ml	NAD
1 ml	NBT
0.5 ml	PMS

Xanthine dehydrogenase

Stain Buffer:

50 ml	0.05M Tris-HCl, pH 7.5
400 mg	hypoxanthine
1 ml	NBT
1 ml	NAD
0.5 ml	PMS
500 mg	KOH

CHEMICALS DESCRIBED IN THIS HANDBOOK

A. Staining Chemicals	Catalogue No.	Chemical Company*
Cis-aconitic acid	A-7251	S
ADP (adenosine-5'-diphosphate)	A-6646	S
ATP (adenosine-5'-triphosphate)	A-5394	S
L-alanine	A-7627	S
3-amino-9-ethylcarbazole	A-5754	S
Arsenic acid, sodium salt	A-6756	S
L-aspartic acid	A-9256	S
Benzidine, dihydrochloride	B-3383	S
Catechol	P-370	F
Choline chloride	C-7017	S
O-dianisidine	D-3127	S
2,6-dichlorophenol-indophenol	D-1878	S
N,N-dimethyl-p-phenylenediamine	D-5004	S
Fast Black K Salt	F-7253	S
Fast Blue BB Salt	F-3378	S
Fast Blue RR Salt	F-0500	S
Fast Garnet GBC Salt	F-6504	S
D-fructose-1,6-diphosphate, trisodium salt	752-1	S
(3-[4,5-dimethylthiazol-2]yl-2,5-diphenyltetrazolium bromide) (MTT)	M-2128	S
D-fructose-6-phosphate, disodium salt	F-3627	S
Fumaric acid, disodium salt	F-1506	S
D(+) galactose, anhydrous	G-0625	S
α-D(+) glucose	G-5000	S
α-D-glucose-1,6-diphosphate, potassium salt	G-5750	S
α-D-glucose-1-phosphate, disodium salt	G-7000	S
D-glucose-6-phosphate, monosodium salt	G-7879	S
L-glutamic acid, monosodium salt	G-1626	S
DL-glyceric acid, hemi-calcium salt	G-5626	S
DL-α-glycerophosphate, disodium salt	G-6126	S
Glycyl-L-leucine	G-2002	S
Hydrogen peroxide	H-1009	S
Hypoxanthine	H-9377	S
DL-isocitric acid, trisodium salt	I-1252	S
α-ketoglutaric acid, monosodium salt	K-1875	S
L-leucine	L-8000	S
L-leucine-β-naphthylamide, hydrochloride	L-0376	S
L-leucyl-L-alanine	L-9250	S
L-leucyl-glycyl-glycine	L-9750	S
DL-malic acid	M-0875	S
D-mannose-6-phosphate, barium salt	M-8754	S
Menadione	M-5750	S
4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside	M-2133	S
4-methylumbelliferyl acetate	M-0883	S
4-methylumbelliferyl butyrate	M-7759	S
4-methylumbelliferyl-β-D-galactoside	M-1633	S

4-methylumbelliferyl-β-D-glucoside	M-3633	S
α-naphthol	N-1000	S
α-naphthyl acetate	N-8505	S
β-naphthyl acetate	N-6875	S
α-naphthyl acid phosphate, monosodium salt	N-7000	S
β-naphthyl acid phosphate, sodium salt	N-7375	S
β-nicotinamide adenine dinucleotide (NAD)	N-0632	S
β-nicotinamide adenine dinucleotide, reduced form (NADH), disodium salt	N-8129	S
β-nicotinamide adenine dinucleotide phosphate (NADP), sodium salt	N-0505	S
β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)	N-1630	S
Nitro blue tetrazolium (NBT)	N-6876	S
Phenazine methosulfate (PMS)	P-9625	S
6-phosphogluconic acid, trisodium salt	P-6888	S
Phosphoenol pyruvate, trisodium salt	P-7002	S
Polyvinylpyrrolidone (PVP-40)	PVP-40	S
Potassium iodide	P-8256	S
Pyridoxal 5'-phosphate	P-9255	S
Riboflavin	R-4500	S
(-) shikimic acid	S-5375	S
Sodium azide	S-2002	S
Sodium bicarbonate	S-8875	S
D-sorbitol	S-1876	S
Succinic acid	S-2378	S
Sucrose	S-9378	S
Thiourea	T-7875	S
2,3,5-triphenyl tetrazolium chloride	T-8877	S
Tryptamine hydrochloride	T-9628	S
Venom, snake (Crotalus viridis helleri)	V-7752	S

B. Chemicals for Buffers	Catalogue No.	Chemical Company*
Acetic acid, glacial	A-38	F
Boric acid	A-73	F
Citric acid	C-0759	S
Ethylenediaminetetraacetate, disodium	S-311	F
DL-histidine, HCℓ	H-7875	S
Lithium hydroxide	L-4256	S
Sodium acetate	S-209	F
Sodium phosphate, dibasic, anhydrous	S-0876	S
Sodium phosphate, monobasic, anhydrous	S-0751	S
Trizma base	T-1503	S

C. Misc. Chemicals	Catalogue No.	Chemical Company*
Acetone	A-19B	F
N,N-dimethylformamide	D-133	F
Ethanol (95%)	-	-
Magnesium chloride	M-33	F
Magnesium sulfate	M-65	F
Maleic anhydride	M-0625	S
Methanol	A-408B	F
Potassium chloride	P-217	F
Potassium hydroxide	P-250	F
Sodium hydroxide	S-318	F
Starch	Lot 375-2	C
Starch	Lot 392	E

D. Enzymes	Catalogue No.	Chemical Company*
Aldolase	A-1893	S
Glucose-6-phosphate dehydrogenase	G-7877	S
Glyceraldehyde-3-phosphate dehydrogenase	G-0763	S
Hexokinase	H-5500	S
Isocitric dehydrogenase	I-2002	S
L-Lactic dehydrogenase	L-2500	S
Malic dehydrogenase	M-7508	S
Peroxidase, crude	P-8000	S
Phosphoglucose isomerase	P-5381	S

*Note: The chemical companies include: (S) Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178, USA; (F) Fisher Scientific Limited, 112 Colonnade Road, Ottawa, Ontario, K2E 7L6; (C) Connaught Laboratories Limited, Willowdale, Ontario, Canada and; (E) Electrostarch Co., P.O. Box 1294, Madison, Wisconsin, 53701, USA. The use of chemicals from the above companies does not constitute an official endorsement or approval from the Dept. of Environment.

APPENDIX III

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